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14. ABSTRACT
Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons in the nigrostriatal pathway resulting in significant motor dysfunction. The pathology of PD is mimicked by exposure to 1-methyl-4-phenyl-1,2,3,tetrahydropyridine (MPTP) or the pesticide rotenone. These neurotoxins inhibit complex I of the mitochondrial respiratory chain resulting in the production of reactive oxygen species (ROS) and increased cytosolic calcium. We hypothesize that ROS promotes opening of the mitochondrial permeability transition pore which triggers the death pathway. In parallel, increases in cytosolic calcium leads to oxidative stress and activation of c-Jun-NH2-terminal kinase (JNK). JNK/c-Jun signaling augments activation of the mitochondrial apoptotic cascade by suppressing Bcl-2 pro-survival signals via phosphorylation of Bcl-2 or transcription of the BH3-only, Bcl-2 antagonist Bim. The interactions between the oxidative stress pathway, the JNK/c-Jun signaling cascade, and the mitochondrial apoptotic machinery ultimately determine the fate of dopamine neurons. We will utilize primary ventral mesencephalic cultures obtained from E15 embryonic rats to investigate our hypothesis. The data obtained should lead to the identification of promising therapeutic strategies to slow or halt the dopaminergic neurodegeneration that occurs during progression of PD.

15. SUBJECT TERMS
neurodegeneration, apoptosis, dopamine neurons, MPTP, rotenone, intrinsic death pathway

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Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons in the nigrostriatal pathway resulting in significant motor dysfunction. The pathology of PD is mimicked *in vitro* and *in vivo* by exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or the pesticide rotenone. These neurotoxins are believed to induce dopaminergic neuronal death via apoptosis, a form of programmed cell death executed by members of the caspase family of cysteine proteases. The signal transduction mechanisms that regulate neurotoxin-induced apoptosis of dopamine neurons are presently unclear. We hypothesize that the neurotoxins, MPTP and rotenone, inhibit complex I of the mitochondrial respiratory chain resulting in the production of reactive oxygen species (ROS) and increased cytosolic calcium. We hypothesize that generation of ROS promotes opening of the mitochondrial permeability transition pore which triggers Bax translocation to mitochondria, cytochrome C release, and caspase activation. In parallel, cytosolic calcium activates neuronal nitric oxide synthase leading to the production of nitric oxide and peroxynitrite. The resulting oxidative stress stimulates c-Jun-NH_2-terminal kinase (JNK) and the transcription factor c-Jun. JNK/c-Jun signaling augments activation of the mitochondrial apoptotic cascade by suppressing Bcl-2-mediated pro-survival signals via JNK-dependent phosphorylation of Bcl-2 or by c-Jun-dependent transcription of the BH3-only, Bcl-2 antagonist Bim. The interactions between the oxidative stress pathway, the JNK/c-Jun signaling cascade, and the mitochondrial apoptotic machinery ultimately determine the fate of the dopamine neuron. We have utilized primary ventral mesencephalic cultures obtained from E15 embryonic rats as a neuronal cell model to investigate our hypothesis. Since dopaminergic neurons make up a very small percentage (< 5%) of these cultures, we have established a number of immunocytochemical probes to study neurotoxin-induced death signaling events in tyrosine hydroxylase-immunoreactive (dopaminergic) neurons.

Specific Aim 1. To investigate the role of the mitochondrial (intrinsic) death pathway in the apoptosis of primary dopamine neurons induced by the neurotoxins MPP+ and rotenone.

1. A. Determine if the intrinsic initiator caspase-9 and the executioner caspase-3 are activated in primary dopamine neurons following incubation with neurotoxins. Aim #1 A. is complete. Rat ventral mesencephalic cultures are well established in the lab. Dopamine neurons are identified by either fixing and staining the cultures with antibodies to detect tyrosine hydroxylase (TH, mouse monoclonal or polyclonal (1:1000) Pel-Freez Biologicals followed by anti-mouse or anti-rabbit Cy3 secondary antibodies) or cultures are transfected by gene-gun at day 4 with a plasmid encoding a 9.0 kb TH promoter region coupled to GFP (provided by Dona Chiraishi, Duke University Medical Center). Cy3-labeled fixed cells or GFP-labeled live cells are analyzed using a Zeiss Axioplan 2 microscope equipped with a Cooke Sensicam deep-cooled CCD camera and Slidebook image analysis software for digital deconvolution (Intelligent Imaging Innovations, Inc.).

Both detection methods for dopamine neurons were used to examine the toxicity of MPP+. The effects of MPP+ were determined to be dose and time-dependent. Maximal death was found between 10-100 micomolar. Using antibodies that specifically recognize the active (cleaved) forms of caspase-9 (rabbit polyclonal, rat-specific, Cell Signaling Technology) or caspase-3 (rabbit polyclonal, Promega), and DAPI to distinguish normal vs. apoptotic nuclei, it appears that most of the dopamine neurons undergo apoptosis after MPP+ treatment. But whether or not all of the cells that have detached after 24 hr of MPP+ treatment died by apoptosis is not clear. We are currently examining shorter periods of time after MPP+ treatment and trophic factor withdrawal (an inducer of intrinsic apoptosis) to determine morphological characteristics of the TH neurons that are dying.

1. B. Effects of a caspase-3 selective inhibitor on the apoptosis of primary dopamine neurons induced by

neurotoxin treatment. Although this aim has been completed, the results have raised questions as to whether apoptosis is the only mechanism of cell death after MPP+ treatment. Rat ventral mesencephalic cultures were treated with MPP+ or subjected to trophic factor withdrawal in the absence or presence of the cell permeable, caspase-3 selective inhibitor, z-DEVD-FMK (Calbiochem). Cultures were fixed and stained with anti-TH antibodies to identify dopaminergic neurons and DAPI to distinguish normal vs. apoptotic nuclei. Caspase -3 inhibitors blocked cell death by both MPP+ and trophic factor withdrawal, but the inhibition of cell death was not complete. So the question remains as to whether some cells die by a non-caspase dependent mechanism and is so, what is the pathway? and why do certain TH neurons die by caspase-dependent mechanism and others die by caspase-3 independent mechanisms?

1. C. Effects of a caspase-9 selective inhibitor on caspase-3 activation and apoptosis induced by neurotoxins. This aim has been carried out. Rat ventral mesencephalic cultures were treated with MPP+ or subjected to trophic factor withdrawal (as described in 1B) in the absence or presence of the cell permeable, caspase-9 selective inhibitor, z-LEHD-FMK (Calbiochem). Cultures were fixed and stained with anti-TH antibodies to identify dopaminergic neurons and DAPI to distinguish normal vs. apoptotic nuclei. The caspase 9 inhibitors blocked cell death to a similar extent as the caspase 3 inhibitors indicating that most of the apoptotic death was mediated by the intrinsic mitochondrial pathway. But again, the blockade of cell death was incomplete, raising the possibility that other death pathways are involved.

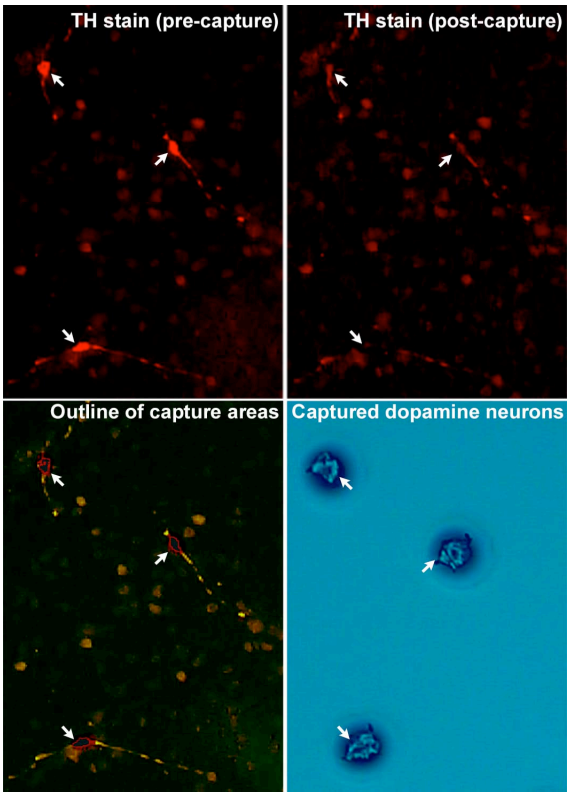
1. D. Determine if Bax translocates to mitochondria and if cytochrome C is released from mitochondria following neurotoxin exposure. In the past year we have established a procedure for measuring Bax translocation and activation in primary cultured neurons and this technique is currently being adapted for dopamine neurons. We obtained a plasmid encoding an amino-terminal GFP fusion protein of human Bax_a from Dr. R. J. Youle (NINDS, NIH). Neurons were transfected with Bax-GFP via particle-mediated gene transfer using a Helios gene gun (Biorad). At 48 h post-transfection, cells were either maintained in control medium or switched to apoptotic medium. After a further 4 h incubation, neurons were fixed in paraformaldehyde and nuclei were stained with Hoechst dye. The localization of GFP or GFP-Bax_a and nuclear morphology were examined by fluorescence microscopy. GFP displayed a diffuse distribution over the entire cell body in neurons cultured in either control or apoptotic medium. In contrast, the localization of GFP-Bax_a changed from a diffuse pattern in control medium to a punctate distribution in neurons cultured in apoptotic medium. To confirm that the punctate distribution of GFP-Bax_a corresponded to its translocation to mitochondria, neurons were stained for the integral mitochondrial membrane protein, cytochrome c oxidase subunit IV (COX IV). In control medium, there was little specific overlap between the localization of GFP-Bax_a and COX IV. While in apoptotic medium, GFP-Bax_a showed substantial co-localization with COX IV. Examination of the kinetics of GFP-Bax_a translocation revealed a rapid movement of the expressed fusion protein to mitochondria upon induction of apoptosis. In control medium, less than 30% of the CGNs expressing GFP-Bax_a showed a mitochondrial localization of the fusion protein. Within 1 h of an apoptotic stimulus, a significant increase in mitochondrial GFP-Bax_a was detectable. After 4 h of incubation in apoptotic medium, approximately 70% of the transfected neurons demonstrated a mitochondrial distribution of GFP-Bax_a. We next analyzed the activation of the endogenous Bax protein by immunostaining neurons with a monoclonal antibody that specifically recognizes the active Bax conformation (clone 6A7). Neurons maintained in control medium showed little-to-no detectable 6A7 staining, whereas neurons in apoptotic medium showed active Bax immunoreactivity. Moreover, the 6A7 staining often coincided with condensed and/or fragmented chromatin. We have begun similar studies in rat ventral mesencephalic cultures. The biggest limitation has been the number of dopamine neurons that get transfected with the Bax-GFP plasmid. Increasing the transfection of dopamine neurons remains one of the highest priorities in the lab. We are currently examining new lipid transfection reagents from Qiagen and we are constructing lenti-viral vectors for use in dopaminergic neuronal cultures. See below.

Specific Aim 4. To evaluate the effects of glial cell-derived neurotrophic factor (GDNF) on neurotoxin-induced generation of reactive oxygen and nitrogen species, activation of JNK/c-Jun, and mitochondrial death signaling.

4. C. Effects of GDNF on neurotoxin-induced activation of the mitochondrial death pathway. VM cultures were incubated with either MPP+ or subjected to trophic factor withdrawal in the absence or presence of increasing concentrations of GDNF. We then assessed an early step in the intrinsic apoptotic cascade, mitochondrial membrane depolarization. In these experiments, dopamine neurons were identified by transfecting the cultures with a GFP-TH reporter using the Helios gene-gun described above. At 48 h post-transfection, approximately 10 to 50 GFP-positive neurons were apparent on each coverslip and 100% of these cells stained positively with an antibody against TH.

To measure mitochondrial membrane potential, cells were washed with Ringer's solution containing 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 5 mM glucose, pH 7.4 and then loaded with 500 nM of tetramethylrhodamine ethyl ester (TMRE) for 30 min at room temperature. TMRE fluorescence was excited at 545 nm usin g excitation light provided by a HBO 100 W lamp in a computer-controlled filter wheel. Emitted fluorescence light was reflected through a 590 nm long-pass filter to the camera. Changes in mitochondrial membrane potential were monitored in single dopamine neurons over time. Initial results show that incubation with MPP+ induced a rapid (within 3 hr) collapse of mitochondrial membrane potential which was prevented by pretreatment with GDNF. The effects of GDNF were antagonized almost completely by the ERK pathway inhibitor, PD98059. Inhibitors of oxidative stress (glutathione), the classical mitoPTP (cyclosporin A), or a Ca^{2+}-triggered permeability transition (2-APB), were also effective at blocking mitochondrial depolarization induced by MPP+. Collectively, these data suggested that GDNF inhibits multiple pathways that induce mitochondrial depolarization of dopaminergic neurons. GDNF appears to blocks both the classical, cyclosporin A-sensitive permeability transition pore and a Ca^{2+}- triggered permeability transition activated by complex I inhibition.

The protection by GDNF required pretreatment of the neurons (8 hr) prior to addition of MPP+. We hypothesize that GDNF counteracts the changes in expression of pro- and anti-apoptotic Bcl-2 family members induced in dopamine neurons by exposure to MPP+. We will utilize Affymetrix analysis to measure changes in the gene expression of primary dopaminergic neurons exposed to MPP+ in the absence or presence of GDNF. Towards this goal, we have spent considerable effort developing laser capture microdissection methods for isolating dopamine neurons from ventral mesencephalic cultutres. We developed an ultra-rapid TH+ staining procedure that allows for the identification of dopamine neurons within 20 minutes (see figure below). Left upper panel shows differential staining for TH with immunoreactive dopamine neurons indicated by the arrows. Following TH staining, dopamine neurons are captured by LCM using an Arcturus AutoPix ^{TM} LCM instrument indicated by the loss of cell bodies in upper right panel and lower left panel (white arrows). The captured dopamine neurons are shown on the surface of the harvest cap (lower tight panel). RNA has been isolated from the captured cells using the RiboAMP RNA amplification kit provided by Arcturus. The quality and quantity of RNA is analyzed with the NanoDrop ND-1000 instrument available at the UCHSC microarray core. cDNA synthesized from the isolated RNA will be used for PCR and Affymetrix analysis. We have performed PCR analysis of tyrosine hydroxylase in isolated dopamine neurons and non-dopamine neurons and the procedure was successful with the capture of 100 dopamine neurons.. It is possible to capture 100-200 cells within an hr.



In the next year, Affymetrix analysis will be carried out by the UCHSC microarray facility. Briefly, samples (in quadruplicate, control dopamine neurons in the absence and presence of GDNF, neurons subjected to MPP+ in the absence and presence of GDNF) will be loaded onto a rat GeneChip® and hybridized overnight in the Affymetrix GeneChip® Hybridization Oven. The chips will be washed and stained in the GeneChip® Fluidics Station 450 and then scanned on the Affymetrix GeneChip® Scanner 3000. Data will be analyzed by running pair wise comparison analysis (control vs. experimental) using the Affymetrix GCOS data analysis software to identify genes upregulated/downregulated in cells undergoing death and in the absence or presence of GDNF. All of the procedures including data analysis will be carried out with Dr. Bifeng Gao, the director of the core facility.

Key Research Accomplishments

The key research accomplishments this year were to finish most of aim #1 and aim #4 and to continue to develop live-cell imaging techniques to explore death pathways in dopamine neurons. We have developed laser capture methods to isolate dopamine neurons from rat ventral mesencephalic neurons so that we can identify gene targets of GDNF.

Reportable Outcomes

Manuscripts

1. **Heidenreich KA** and DA Linseman**.** Myocyte enhancer factor-2 (MEF2) transcription factors in neuronal differentiation and survival. Mol. Neurobiol. 29: 155-166, 2004.

2. McClure ML, DA Linseman, CT Chu, RJ Bouchard, TA Laessig, SS Le, and **KA Heidenreich**. Neurotrophins and death receptors regulate autophagic death in cerebellar Purkinje neurons. J. Neuroscience 24: 4498-4509, 2004.

3. Linseman, DA*, BD Butts*, TA Precht, RA Phelps, SS Le, TA Laessig, RJ Bouchard, ML McClure, and KA Heidenreich. Glycogen synthase kinase-3b phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. *co-first authors J. Neuroscience 24: 9993-10002, 2004.

4. Precht, TA, RA Phelps, DA Linseman, BD Butts, RJ Bouchard, SS Le, TA Laessig, and **KA Heidenreich**. Bax translocation to mitochondria is triggered by permeability transition pore opening in cerebellar granule neurons undergoing apoptosis. Cell Death and Differentiation, 12: 255-265, 2005.

5. Zimmermann, AK, FA Loucks, SS Le, BD Butts, M McClure, RJ Bouchard, **KA Heidenreich,** DA Linseman. Bcl-2 interacting mediator of cell death (Bim) induces cerebellar granule neuron apoptosis via a mechanism that is independent of Bcl-2 antagonism. J. Neurochem.94: 22-36, 2005.

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7. Le, SS, FA Loucks, H Udo, S Richardson-Burns, RA Phelps, RJ Bouchard, H Barth, K Aktories, KL Tyler, ER Kandel, **KA Heidenreich**, and DA Linseman. Inhibition of Rac GTPase triggers a c-jun -and Bim-dependent mitochondrial apoptotic cascade in cerebellar granule neurons. J. Neurochem. 94: 1025-1039, 2005.

Abstracts

1. Linseman DA, Butts BD, Precht TA, Phelps RA, Le SS, Laessig TA, Bouchard RJ, and **Heidenreich KA** Glycogen synthase kinase-3beta phosphorylates Bax and triggers its localization to mitochondria during neuronal apoptosis. Keystone Symposia – Apoptosis in Biochemistry and Structural Biology. 2004

2. Loucks FA, Zimmermann AK, Le SS, Bouchard RJ, Laessig TA, **Heidenreich KA**, and Linseman DA Antagonism of Bcl-2 reveals oxidative stress-dependent and –independent pathways for BH3-only-mediated neuronal apoptosis. Keystone Symposia – Apoptosis in Biochemistry and Structural Biology, 2004.

3. Hudson HR, Butts BD, DA Linseman, SS Le , and **KA Heidenreich.** Proteasome inhibition elicits a biphasic effect on neuronal apoptosis via differential regulation of pro-survival and pro-apoptotic transcription factors. Rocky Mountain Regional Neuroscience Meeting, June 2004.

4. Zimmermann AK, Loucks FA, Le SS, Bouchard RJ, Laessig TA, **Heidenreich KA**, and Linseman DA Bcl-2 interacting mediator of cell death (Bim) induces cerebellar granule neuron apoptosis via a mechanism that is independent of Bcl-2 antagonism. Rocky Mountain Regional Neuroscience Meeting, June 2004.

5. Chen, C, DA Linseman, BD Butts, RJ Bouchard, ML McClure, **KA Heidenreich**. GDNF protects primary dopamine neurons from MPP+induced toxicity by blocking diverse pathways to mitochondrial depolarization. Society for Neuroscience , San Diego, 2004.

6. Butts BD, HR Hudson, DA Linseman, SS Le , and **KA Heidenreich.** Proteasome inhibition elicits a biphasic effect on neuronal apoptosis via differential regulation of pro-survival and pro-apoptotic transcription factors. Society for Neuroscience , San Diego, 2004.

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8. Chen, C, DA Linseman, BD Butts, RJ Bouchard, ML McClure, **KA Heidenreich**. GDNF protects primary dopamine neurons from MPP+induced toxicity by blocking diverse pathways to mitochondrial depolarization. Front Range Neuroscience Meeting, Fort Collins, CO, 2004.

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10. Precht TA, Phelps RA, Linseman DA, Butts BD, Bouchard RJ, and **Heidenreich KA.** The mPTP triggers Bax translocation to mitochondria during neuronal apoptosis. Keystone Symposia – Cellular Senescence and Cell Death, Keystone, CO, 2005.

11. Chen C, Linseman DA, Butts BD, Bouchard RJ, McClure ML, and **Heidenreich KA**. GDNF protects primary dopaminergic neurons from MPP+-induced toxicity by blocking diverse pathways to mitochondrial depolarization. Keystone Symposia – Cellular Senescence and Cell Death, Keystone, CO, 2005.

12. Butts BD, Hudson HR, Linseman DA, Le SS, and **Heidenreich KA**. The role of the proteasome in neuronal apoptosis. Keystone Symposia – Cellular Senescence and Cell Death, Keystone, CO, 2005.

13. Zimmermann AK, Loucks FA, Bouchard RJ, **Heidenreich KA**, and Linseman DA. Distinct inhibitors of Bcl-2/Bcl-X(L) function elicit a common glutathione-sensitive pathway of neuronal death. Keystone Symposia – Cellular Senescence and Cell Death, Keystone, CO, 2005.

14. Linseman, DA, C Chen, BD Butts, RJ Bouchard, **KA Heidenreich**. GDNF protects primary dopamine neurons from MPP+induced toxicity by inhibiting multiple pathways of mitochondrial depolarization. 16th International Congress on Parkinson's Disease, 2005.

15. Zimmermann, AK, FA Loucks, RJ Bouchard, **KA Heidenreich**, and DA Linseman. Role of complex III and ANT in GSH-sensitive neuronal death induced by inhibitors of Bcl-2/x(L). Soc. Neuroscience, 2005.

16. Precht TA, RA Phelps, DA Linseman, BD Butts, RJ Bouchard, and **KA Heidenreich**.. The mPTP Triggers Bax Translocation to Mitochondria During Neuronal Apoptosis. Rocky Mountain regional Neuroscience Group, Denver, CO, May 2005. poster award

17. Zimmermann, AK, FA Loucks, RJ Bouchard, **KA Heidenreich**, and DA Linseman. Role of complex III and ANT in GSH-sensitive neuronal death induced by inhibitors of Bcl-2/x(L). Rocky Mountain Regional Neuroscience Group, Denver, CO, May 2005. poster award

18. Zimmermann, AK, FA Loucks, RJ Bouchard, **KA Heidenreich**, and DA Linseman. Distinct inhibitors of Bcl-2/X(L) function elicit a common glutathione-sensitive pathway of neuronal death via oxidation of adenine nucleotide translocase. American Society for Neurochemistry 36th Annual meeting, 2005.

19. Zimmermann, AK, FA Loucks, RJ Bouchard, **KA Heidenreich**, and DA Linseman. Role of complex III and ANT in GSH-sensitive neuronal death induced by inhibitors of Bcl-2/x(L). Society for Neuroscience, 2005.

Invited talks- Heidenreich

2004	Neuroscience program, University of Michigan, Ann Arbor, "Molecular Mechanisms of Neuronal Death"
2004	Department of Pathology, University of Pittsburgh, Pittsburgh, PA, " Molecular Mechanisms of Neuronal Cell Death"
2005	Invited symposium speaker, Gordon Conference on IGFs, "The Neuroprotective Actions of IGFs, Ventura, CA.

The scope of research over the last year has focused on Specific Aims #1 and #4 of our original research proposal. We have also spent effort in developing laser capture microdissection methods for isolating dopamine neurons from rat ventral mesencephalic neurons. We were successful in obtaining a VA equipment grant for a Veritas laser capture microdissection instument. Our findings were presented at numerous national and international meetings and have resulted in numerous publications.